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Production of Hybrid 16-Membered Macrolides by Expressing Combinations of Polyketide Synthase Genes in Engineered *Streptomyces fradiae* Hosts

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Summary

Combinations of the five polyketide synthase (PKS) genes for biosynthesis of tylosin in *Streptomyces fradiae* (*tyl*G), spiramycin in *Streptomyces ambofaciens* (*srm*G), or chalcomycin in *Streptomyces bikiniensis* (*chm*G) were expressed in engineered hosts derived from a tylosin-producing strain of *S. fradiae*. Surprisingly efficient synthesis of compounds predicted from the expressed hybrid PKS was obtained. The post-PKS tailoring enzymes of tylosin biosynthesis acted efficiently on the hybrid intermediates with the exception of TylH-catalyzed hydroxylation of the methyl group at C14, which was efficient if C4 bore a methyl group, but inefficient if a methoxyl was present. Moreover, for some compounds, oxidation of the C6 ethyl side chain to an unprecedented carboxylic acid was observed. By also expressing *chmH*, a homolog of *tylH* from the chalcomycin gene cluster, efficient hydroxylation of the 14-methyl group was restored.

Introduction

The 16-membered macrolides (16-MM) are an important class of antibiotic that are used in human and veterinary medicine. They are structurally related to the better-known 14-membered macrolides (see Figure 1). Biosynthesis begins with assembly of a macrolactone ring by a modular polyketide synthase (PKS) [1]. Six different 16-membered macrolactone ring structures are known, from which more than 200 antibiotics are derived through different post-PKS tailoring reactions [2]. The PKSs for all known 16-MMs consist of five large polypeptides with a conserved modular organization. The macrolactone product is determined by the specificities of catalytic domains within each module. Figure 2 shows the domain organization for the tylosin (*tyl*), spiramycin (*srm*), and chalcomycin (*chm*) PKSs, as well as the macrolactone product of each enzyme, tylactone, platenolide, and chalcolactone, respectively. Both the biosynthesis of tylosin and the involvement of genes in the tylosin cluster have been studied extensively [3–14]. Less has been reported on spiramycin biosynthesis, although the PKS genes were cloned and sequenced some time ago [15, 16]. We have recently characterized the chalcomycin gene cluster [17] and found some unusual features with respect to formation of the 2,3-*trans* double bond.

This work focused on engineering the biosynthesis of 16-MMs related to midecamycin A1 (see Figure 1) to introduce a chemical handle for attachment of side chains on the left side of the molecule that could potentially enhance antibiotic activity against macrolide-resistant strains. Three macrolide binding regions of the 50S ribosomal subunit are associated with antibacterial activity (1) near the residue corresponding to adenosine 2058 of the 23S rRNA (*Escherichia coli* numbering) in domain V, (2) in the peptidyl transferase active site of domain V, and (3) in domain II (hairpin 35) of the 23S rRNA [18–26]. All macrolides interact with the A2058 region and methylation of this residue substantially reduces binding and confers high-level resistance [22, 23, 27]. Midecamycin A1 and other 16-MMs that have an acyl group extending from the disaccharide component can reach into the peptidyl transferase site and inhibit its activity [20, 21], providing some restoration of potency against resistant strains [28]. Ketolides such as telithromycin, derived from erythromycin by attachment of a side chain, are active against some macrolide-resistant pathogens such as *Streptococcus pneumoniae* [29]. The side chain interacts with nucleotides in the domain II region [26, 29]. Most 16-MMs are not amenable to chemical attachment of side groups for domain II binding, and the PKS engineering described here was aimed at installing such a handle. Specifically, we engineered hybrid 16-MMs with a 14-hydroxymethyl group that should allow attachment of side chains via cyclic carbamates between the 14-hydroxymethyl group and an unhindered 12,13 double bond, similar to chemistry used in the synthesis of telithromycin [29].

To produce 16-MMs derived from hybrid macrolactones consisting of chalcolactone and either platenolide or tylactone, the first two genes of the *chm* PKS cluster, encoding the loading and three extender modules, were expressed along with the last three from either the *srm* or *tyl* PKS, encoding four extender modules, in specially constructed *S. fradiae* hosts in which the native *tyl* PKS-encoding genes were either deleted or inactivated. In these strains, all or most of the *tyl* biosynthesis genes involved in post-PKS processing were present. Introduction of hybrid PKSs into these hosts resulted in efficient production of novel structures derived from the predicted macrolactone. However, some of these molecules were not efficiently hydroxylated on the 14-methyl group by the TylH hydroxylase. To correct this, a homologous hydroxylase gene from the chalcomycin producer was introduced.

Results and Discussion

Expression of a Hybrid Chalcomycin-Spiramycin PKS Operon in *S. fradiae* Yields a Series of Novel Compounds

A pSET152-derived vector carrying the *chmGI-II-srmGIII-V* genes as a single operon was introduced into

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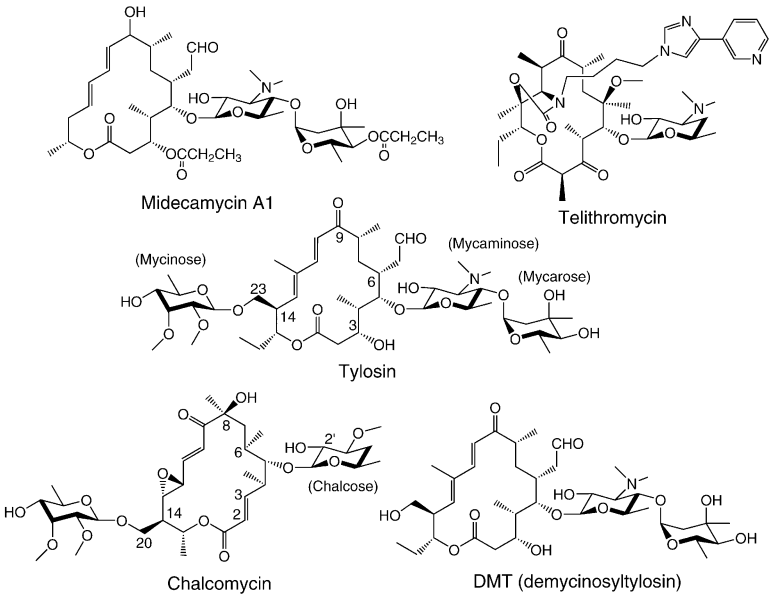

Figure 1. Structures of Macrolide Antibiotics and Intermediates Relevant to the Development of New Antibiotics Using PKS Engineering

S. fradiae K159-1/pKOS244-017a, in which the tylosin PKS genes had been deleted [30], and in which the genes *fkbGHIJK* for the methoxymalonyl-ACP precursor from *Streptomyces hygroscopicus* had been integrated at the pSAM2 *att* site [31]. The methoxymalonyl-ACP precursor is a required substrate for platenolide PKS module 5 [31]. The hybrid PKS operon was expressed from *tylGIp*, which was previously shown to be a strong promoter in *S. fradiae* [30]. To facilitate construction of the hybrid operon, the C-terminal interaction domain [32] of the second chalcomycin PKS polypeptide (ChmGII) was replaced with that of the spiramycin PKS (SrmGII) at a unique HindIII site. Transconjugants were patched on R5 agar, and plugs were screened for bioactivity on *Micrococcus luteus*. After 7 days growth, most plugs gave large zones of inhibition. LC-MS analysis of an ethyl acetate extract of the agar under these zones showed a major peak with a molecular mass of *m/z* 714.85 $[MH]^+$. The methanol adduct was also observed, which is generally diagnostic of an aldehyde function. Upon purification, it was identified by NMR spectrometry as 4″-despropionyl-14-methylniddamycin (DPMN, Figure 3), a compound derived from 14-methylplatenolide. After shake flask fermentation of one of the isolates (*S. fradiae* K232-192), LC-MS analysis of whole broth revealed three major compounds: *m/z* 585.68 $[MH]^+$, *m/z* 714.85 $[MH]^+$, and *m/z* 730.85 $[MH]^+$, in order of abundance, as well as a low level of a compound of *m/z* 905.05 $[MH]^+$. Surprisingly, only DPMN and the *m/z* 905.05 $[MH]^+$ compound were extracted into ethyl acetate, so solid-phase extraction of the spent aqueous phase was used to recover and purify the *m/z* 585.68 $[MH]^+$ compound. NMR analysis revealed its structure to be that of the acidic compound, 6-carboxymethyl-6-desethyl-5-*O*-mycaminosyl-14-methylplatenolide (CDMP, Figure 3), in which the C6 ethylaldehyde had

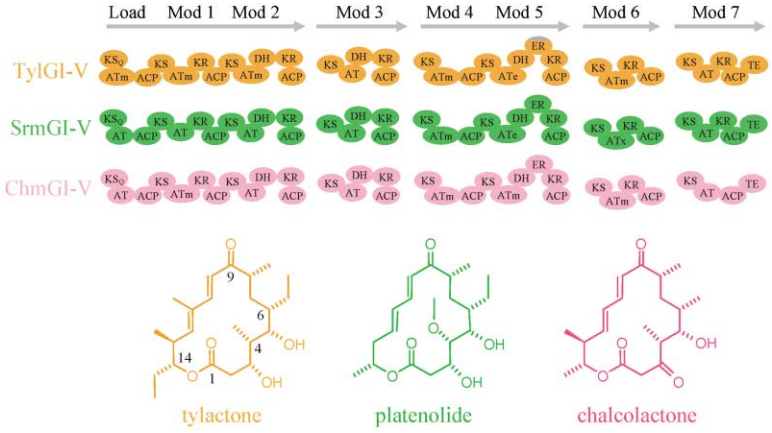

Figure 2. Comparison of the Domain Structure for the Tylosin, Spiramycin, and Chalcomycin PKSs and the Macrolactone Produced by Each

At the top is shown the organization of seven modules on five polypeptides, which is universally conserved among the 16-MMs. Below that the organization of domains within each module is shown for the three PKSs: starter module decarboxylation (KSQ); acyl transferase with specificity for malonyl, methylmalonyl, ethylmalonyl, or methoxymalonyl extender units (AT, ATm, ATe, and ATx, respectively); acyl carrier protein (ACP); ketosynthase (KS); ketoreductase (KR); dehydratase (DH); enoylreductase (ER); and thioesterase macrolactonization (TE). The structures of the PKS products are shown at the bottom with chalcolactone being the structure obtained by expression of the chalcomycin PKS in *S. fradiae* and not necessarily the structure obtained from the PKS in its native context in *S. bikiniensis* [17].

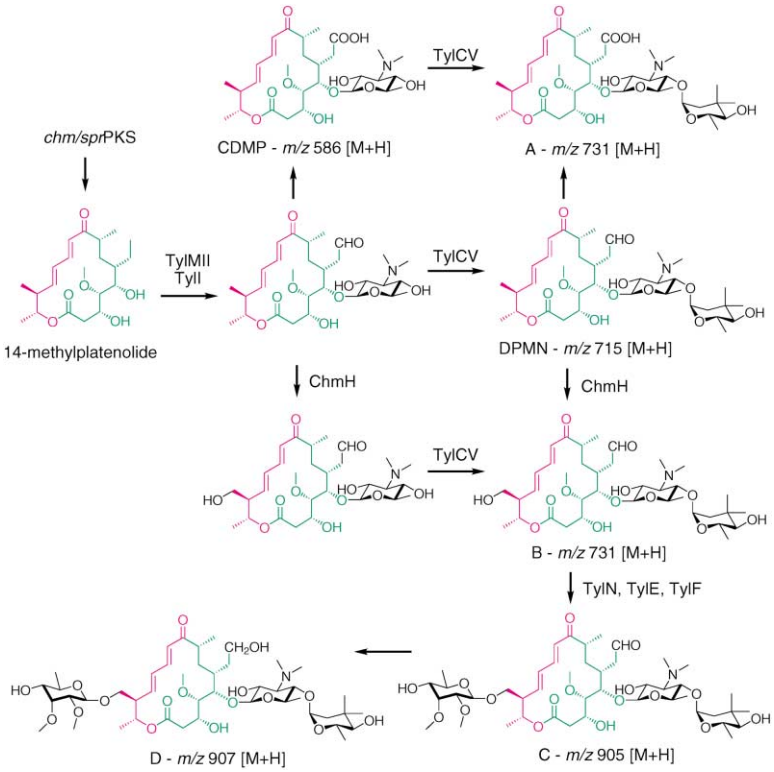

Figure 3. Proposed Post-PKS Tailoring Pathways for the Chalcolactone-Platenolide Hybrid

The portions of the polyketide core derived from the corresponding PKS are indicated using the same color coding as in Figure 2.

been oxidized to the corresponding carboxylic acid, a feature not previously reported for a 16-MM. It was inferred that the *m/z* 730.85 $[MH]^+$ compound was 4′-*O*-mycarosyl-CDMP, i.e., it also had the carboxylic acid (labeled A in Figure 3). The low level of hydroxylation of the C14 methyl group indicates that intermediates derived from the chalcolactone-platenolide hybrid structure are poor substrates for the TylH hydroxylase of the host. Oxidation of the aldehyde to the carboxylic acid may be catalyzed by TylI, which is known to generate the aldehyde through two sequential hydroxylations, or by an unlinked P450 enzyme that happens to recognize the intermediate as a substrate.

Estimation of titers from areas under the peaks at 280 nm compared with a standard curve of purified DPMN, indicated the total polyketide titer was only slightly less than that of tylosin produced by the parent strain under the same fermentation conditions (~2 g/l). Previously, the only report of a hybrid PKS composed exclusively of modules from 16-MM PKSs was the spiramycin PKS in which the loading domain was exchanged with the loading domain from the tylosin PKS, resulting in production of 15-methylspiramycin [16]. Although an engineered PKS often has reduced catalytic efficiency, either because of impaired PKS function through structural distortion or because the unnatural intermediate is a poor substrate for a downstream step, production of polyketides by this hybrid 16-MM PKS was nearly as efficient as tylosin production by the parent strain. The functional compatibility between different 16-MM PKS polypeptides presumably reflects the significant sequence similarity resulting from relatively recent divergence from a common ancestor. Although replacing the C-terminal interaction domain of ChmGII may not have been necessary, since these domains are highly conserved among the 16-MM PKSs, it was clearly not detrimental.

Expression of *chmH* in *S. fradiae* K232-192 Enhances 14-Methyl Hydroxylation

The chalcomycin gene cluster encodes a close homolog of the TylH hydroxylase, designated ChmH [17]. Each has a ferredoxin gene immediately downstream that may be important for activity or specificity. To determine whether the ChmH hydroxylase would accept a chalcolactone-platenolide derivative as a substrate for hydroxylation of the C14 methyl group, the *chmH* gene with its cognate ferredoxin gene was introduced into the host expressing the *chmGI-II-srmGIII-V* hybrid PKS genes using a modified version of the ϕBT1-based integrating vector, pRT802 [33], carrying *tsr* for thiostrepton resistance and *tylGIp* for expression of inserted genes. Introduction of *chmH* had a dramatic affect on the profile of compounds produced by the strain (Figure 4). Although there was still a significant amount of CDMP present, compounds with masses of *m/z* $[MH]^+$ 730.85, 907.04, and a trace of 905.04 were also observed. Since these three compounds could be extracted into ethyl acetate (Figure 4C), they are inferred to be the compounds labeled B, C, and D, respectively, in Figure 3. Although only the structures of DPMN and CDMP were verified by NMR spectrometry, the structures of the other compounds can be inferred from the well-characterized post-PKS tylosin pathway, the ethyl acetate extractability of the compounds, the presence or absence of a methanol adduct (hemiacetal), and the LC-MS data itself

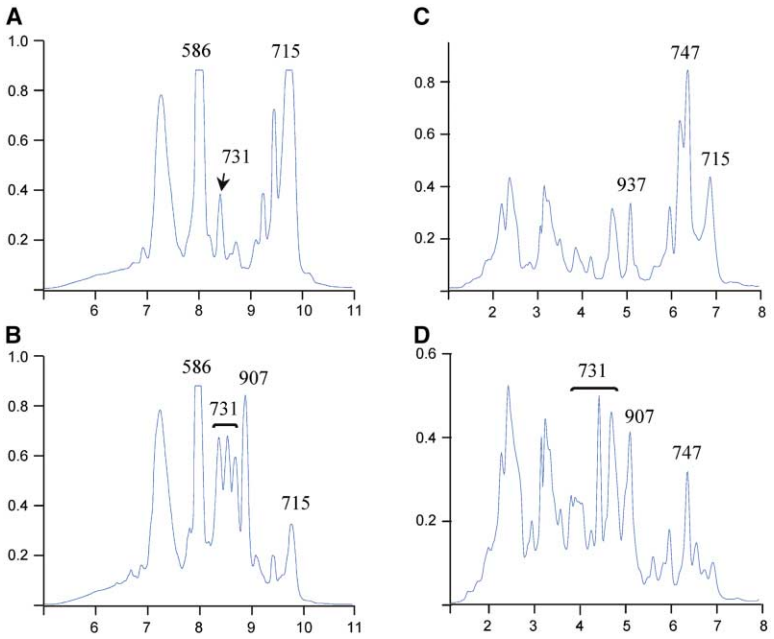

Figure 4. Effect on the Profile of Products Derived from Fermentation of the Strain Expressing the Chalcolactone-Platenolide Hybrid PKS when the *chmH* Gene Is Also Expressed

Shown are the LC-MS traces at 280 nm with the masses of specific peaks of interest indicated above. The x axis indicates the time in minutes from sample injection and the y axis indicates the absorbance at 280 nm. (A) and (B) show products from the strain not containing the *chmH* gene, while (C) and (D) show products from the strain that did contain this gene. (A) and (C) show the LC-MS traces for clarified whole fermentation broth, while (B) and (D) show the traces for products extracted from whole broth into ethyl acetate and the dried residue dissolved in methanol.

(relative retention times and masses). Thus, ChmH can efficiently hydroxylate the 14-methyl group of the intermediates derived from the *chm-srm* hybrid PKS, even though TylH does so very poorly. Figure 3 presents the post-PKS tailoring pathways for the chalcolactone-platenolide hybrid both in the presence or absence of ChmH. It appears that once hydroxylation of the 14-methyl group occurs, the resulting intermediates are resistant to oxidation of the aldehyde.

The first two post-PKS reactions of tylactone are attachment of mycaminose to the C5 hydroxyl by TylMII and oxidation of the C6 ethyl side chain to an aldehyde through two sequential hydroxylations by TylI [4, 6]. All known 16-membered macrolactones have a sugar at the C5 hydroxyl, usually mycaminose, although chalcomycin has chalcose instead [34]. When the macrolactone bears a C6 ethyl side chain, it is always oxidized to an aldehyde. The enzymes for these first two reactions in the tylosin pathway apparently have considerable substrate tolerance, since all the compounds observed here had at least these two post-PKS modifications. Although there is a preferred pathway for the post-PKS reactions in tylosin biosynthesis [4], the order of some steps is flexible and depends on the relative substrate tolerance of the enzymes. For example, a *tylD* mutant of *S. fradiae* produces demycinosyltylosin (DMT) [6], indicating that attachment of the allose sugar does not have to precede attachment of the mycarose sugar.

The significant level of the *m/z* 907 $[MH]^+$ compound produced is believed to result from reduction of the aldehyde of the *m/z* 905 $[MH]^+$ compound to the alcohol. This reaction converting tylosin to tylosin D is known to occur in *S. fradiae*, and the enzyme responsible has been characterized [35]. Apparently, the tylosin analog derived from expression of the *chmGI-II-srmGIII-V* hybrid PKS is a particularly good substrate for this enzyme. Strain K232-192 not expressing *chmH* did not produce detectable *m/z* 907 $[MH]^+$ compound, though it did produce a low level of the *m/z* 905 $[MH]^+$ compound. Perhaps a threshold level of the *m/z* 905 $[MH]^+$ compound is required to induce the reductase activity.

Complementation of a KS1° *S. fradiae* Strain with the First Two Genes of the Chalcomycin PKS Gives a Single Novel Compound

Changing the active site cysteine residue to an alanine in the first keto synthase (KS) domain of the erythromycin PKS blocks synthesis of the product, 6-deoxyerythronolide B, but allows appropriate diketides, as their N-acetylcysteamine thioester derivatives, to be fed to the KS1° host, giving the corresponding macrolactones and showing that modules 2–6 of the PKS are functional in the mutant [36]. When a similar mutation was introduced into the KS1 domain of the *tyl* PKS of a DMT producer of *S. fradiae*, i.e., bearing a *tylD* mutation [5], feeding of the appropriate diketide thioester also restored production of DMT (data not shown), indicating that modules 2–7 in the *tyl* PKS were functional. A synthetic operon containing the *chmGI-II* genes under the control of *tylGIp* were integrated into the chromosome of this *S. fradiae* strain at the ϕC31 *att* site using a derivative of pSET-152 [30]. Although the heterologous ChmGII polypeptide (carrying module 3) can probably interact appropriately with the host TylGIII polypeptide (carrying modules 4 and 5), the C-terminal interaction domain of ChmGII was replaced with the TylGII counterpart to ensure an optimum interaction based on the results with the ChmGI-II-SrmGIII-V hybrid discussed above. The resulting strain (*S. fradiae* KS1°/pKOS342-84) produced antibiotic activity against *M. luteus*, and LC-MS analysis of the fermentation broths revealed a compound of *m/z* 714.88 $[MH]^+$. Isolation and NMR analysis showed the structure to be 12,16-didesmethyl-DMT, in agreement with the prediction. The titer of 12,16-didesmethyl-DMT was approximately 0.5 g/l, indicating that the presence of the inactive TylGI and active TylGII

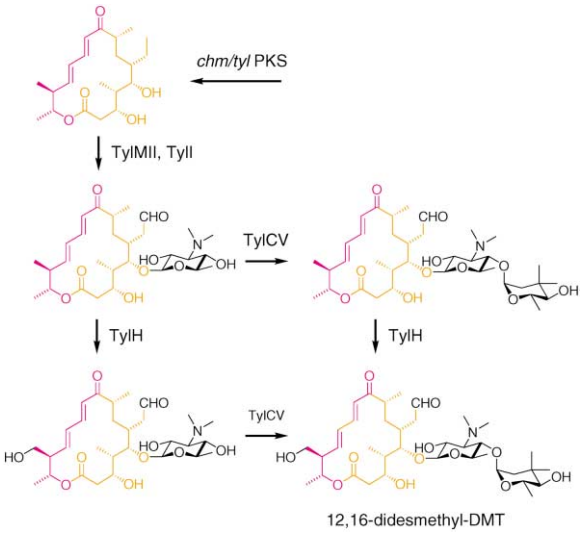

Figure 5. Proposed Post-PKS Tailoring Pathways for the Chalcolactone-Tylactone Hybrid in the *tylD*, KS1° Double Mutant Strain

See text for details.

polypeptides did not significantly interfere with the interaction between ChmGI and either ChmGII or TylGII, which have identical domain organization and should both interact with TylGIII.

Figure 5 shows the post-PKS tailoring pathways leading to the chalcolactone-tylactone hybrid. Since 12,16-didesmethyl-DMT was produced efficiently, the presence or absence of methyl groups on C12 and C16 has little effect on TylH-catalyzed hydroxylation of the 14-methyl group (or the other post-PKS reactions). Although the 14-methyl group of the *chmGI-II-tylGIII-V* hybrid PKS product is efficiently hydroxylated by TylH, neither the product of the *chmGI-II-srmGIII-V* hybrid PKS nor the product of the complete chalcomycin PKS [17] are good substrates for 14-methyl hydroxylation by TylH. This suggests that an oxygen atom on C3 or C4 with a particular position relative to the 14-methyl group may inhibit the TylH hydroxylase. Because the ChmH hydroxylase presumably evolved to function in the presence of this oxygen, it is perhaps not surprising that it can hydroxylate the chalcolactone-platenolide hybrid.

Significance

An important goal of PKS engineering is to generate unique molecules that can be used as starting points for subsequent chemical modification. The 14-hydroxymethyl derivatives produced in this study are examples of potential starting points. These compounds lack the 12-methyl side chain of tylosin and its intermediates and should allow cyclization chemistry on the left side of the molecules equivalent to that employed in ketolide synthesis in the 14-membered macrolide group. It has also been observed previously that the catalytic efficiency of hybrid or mutant PKS enzymes can be significantly reduced. This work shows that coexpression of genes encoding the polypeptides of different, but related, PKSs can give highly efficient production of novel products. The strategy is useful in cases where there are several highly homologous sets of PKS genes available that encode the production of slightly different structures. Thus, use of the *S. fradiae* host strains described here for expression of PKS gene combinations allows production of 16-MMs derived from novel macrolactones. The post-PKS tylosin pathway is generally tolerant of structural variations, although the TylH hydroxylase could not hydroxylate the 14-methyl group of one of the hybrids. In cases where a post-PKS enzyme of the tylosin pathway will not accept the hybrid structure as a substrate, there may be an alternative enzyme that can be recruited from another 16-MM pathway, as was demonstrated for the ChmH hydroxylase.

Experimental Procedures

Strains, Growth Media, and Basic DNA Manipulation

All strains were derived from a tylosin-overproducing *S. fradiae* strain [30]. Deletion of the tylosin PKS genes from the strain was described previously [30]. DNA was delivered into *S. fradiae* strains by conjugative transfer of a mobilizable plasmid from *E. coli* DH5α harboring the RK2-derived helper plasmid pUB307 [30]. The conjugation protocol was as described [37], except that overnight incubation prior to the antibiotic overlay was at 37°C. Plasmids were propagated in *E. coli* DH5α and were constructed using well-established methods [38]. Antibiotic-resistant exconjugants of *S. fradiae* were streaked for single colonies on AS-1 medium containing the appropriate antibiotics. When exconjugants of a nonproducing host were expected to produce a tylosin-related analog, they were patched onto R5 medium and, after 7 days, plugs were screened for their ability to form zones of inhibition on lawns of *Micrococcus luteus* ATCC 9341 growing on Difco medium 11.

Construction of *S. fradiae* Strains

S. fradiae K264-105.2 contains a deletion of the *tylD* gene and a null mutation in KS1 of *tylGI*. Deletion of *tylD* involved sequential double homologous crossing over after introduction of a plasmid carrying regions flanking the gene. The ca. 1.8 kb regions were isolated by PCR using the following oligonucleotides: 5′-GGCATGC CCAGAACCAGTACCCGGTCACATG-3′, with 5′-GCTGCAGCCCAC TCGTGAATCCCGAAGGGAAG-3′, and 5′-CCTGCAGCGTAGTGGG AGCCGATGAAGCCCA-3′, with 5′-GGAATTCCTGACACAGACCGG TCACCGTTCGT-3′. This introduced the unique restriction sites (EcoRI-PstI and PstI-SphI). The PCR products were ligated between the EcoRI and SphI sites of pOJ260 [39] giving pKOS168-106, in which 80% of the *tylD* gene was removed and the reading frame maintained. After conjugation of the *E. coli* donor with the *S. fradiae* K155-3C [30] recipient, apramycin-resistant colonies were obtained, and those with the plasmid integrated at the *tylD* locus were identified by Southern blot hybridization. Growth in the absence of selection gave *S. fradiae* K168-173, which had become apramycin sensitive and produced DMT. The KS1° mutation was introduced by changing the active site cysteine to alanine and introducing a NheI site. This was accomplished using PCR to make two fragments. The first was a 260 bp fragment made using the oligonucleotides 5′-ATGGATCCGCAGCAGCCCGTGT-3′ and 5′-GCTAGCCGCCGTAT CCACGGTCACGG-3′. The second was a 370 bp fragment made using the oligonucleotides 5′-GCTAGCTCGTCGTTGGTGGCGTTG CATCT-3′ and 5′-GCGCATTCCCCAACGCCTGACGAAT-3′. The first fragment was digested with BamHI and NheI and the second fragment with NheI and BsmI. These two pieces were then ligated into BamHI and BsmI digested pKOS264-65, which is pUC19 containing the ca. 6 kb EcoRV/EcoRI fragment from *tylGI*, to generate pKOS325-8. The insert from pKOS325-8 was isolated by digestion with XbaI and PvuII and ligated into pKOS241-52 (derived from pSET152 by cutting with SphI/HindIII, blunting with Klenow Polymerase, and self ligating). This suicide plasmid, designated pKOS264-76, was introduced into *S. fradiae* K168-173 using an *E. coli* donor.

The first and second crossover events were verified by Southern blot hybridization and the strain, K264-105.2 was shown to produce no tylosin-related compounds. However, it did produce DMT when 2(S)-methyl-3(R)-hydroxypentanoyl-N-acetylcysteamine was fed to the strain (M.M., W.P.R., and L.K., unpublished data).

Construction of the host with the tylosin PKS genes deleted (*S. fradiae* K159-1) is described elsewhere [30]. Construction of strain K159-1/pKOS244-017a by introducing a set of genes from the FK520 (ascomycin) gene cluster of *S. hygroscopicus* ATCC 14891 (*fkbGHIJK*) that provide the precursor methoxymalonyl-ACP is also described elswhere [31].

Construction of Vectors for Expression of PKS Genes

pKOS232-189 is an expression vector similar to pSET152 [39], but containing *tylGIp* with a NdeI site at the start of transcription, and a λ cos site for packaging of large constructs. The *tylGIp* was amplified by PCR [30], ligated into the EcoRV site of Litmus38, then isolated as a NdeI/PstI fragment. This fragment, a 3 kb NdeI/SpeI fragment of heterologous PKS sequence, and pKOS159-31 [30] cut with NsiI and SpeI were ligated together to create pKOS232-189.

The integrative expression vector, pRT802, was made using the *attP* site and *int* genes from the phage φBT1 [33]. Multicloning sites were added using a linker made from the following oligonucleotides: 5′-GGCCGCGTCAATTGGCCGTTAATTAACGCATATGGCCTAGGC GAGGCCTGCATGCATCCAAGCTTCG-3′ and 5′-GATCCGAAGCTT GGATGCATGCAGGCCTCGCCTAGGCCATATGCGTTAATTAACGG CCAATTGACGC-3′, to give pKOS231-149. The *tylGIp* was then introduced by digesting the *tylGIp*/Litmus38 plasmid mentioned above with NotI and NdeI and ligating it into pKOS231-149 digested with the same enzymes to create pKOS231-153D. To isolate the ampicillin resistance gene, Litmus28 was digested with HpaI and SwaI. This was ligated into pKOS231-153D digested with BspHI and end-filled with Klenow polymerase to give pKOS231-183D. To isolate the thiostrepton resistance gene, pIJ5719 (C. Khosla, personal communication) was digested with NdeI, end-filled with Klenow Polymerase, and digested with SmaI. This fragment was ligated to EcoRV-digested Litmus38 to give pKOS342-106B. This was digested with BamHI and HindIII to ligate the thiostrepton resistance gene into pKOS231-183D digested with the same enzymes, which created the expression vector pKOS342-108D.

Constructs for Expression of 16-MM PKS Genes in *S. fradiae*

Characterization of the chalcomycin gene cluster and construction of an expression plasmid containing the first two chalcomycin PKS genes, pKOS232-172, are described elsewhere [17]. To replace the C-terminal interaction domain of ChmGII with the corresponding TylGII linker, a short fragment from the 3′ end of *tylGII* was amplified by PCR using the primers 5′-TGAAGCTTCCCGCCACGCTGGTG-3′, 5′-CGTCTAGACAGGGTGTGAAACCG-3′, and pKOS168-190 [30] as the template, which introduced a HindIII site corresponding to the natural site in *chmGII*. The PCR product was digested with HindIII and XbaI and ligated into pKOS232-172 cut with the same enzymes to create pKOS342-82. The insert of this plasmid was excised with NdeI and XbaI and ligated with the vector portion of pKOS232-189 digested with NdeI and SpeI to give pKOS342-84. This plasmid was moved into *E. coli* DH5α/pUB307 and then into *S. fradiae* K264-105.2 by conjugation to give the strain *S. fradiae* K342-84.

A natural, unique HindIII site near the 3′ end of *chmGII* was used to attach the chalcomycin genes to the last three spiramycin PKS genes. The three spiramycin genes were assembled from subclones of cosmid pKC1306 (unpublished Eli Lilly deposit NRRL B21499). A HindIII site was introduced into *srmGII* at the same position as for *chmGII* by PCR amplification using the following upstream primer: 5′-ACACGCTTAAGACTGAAGCTTCCCGCGACCTCGTCTTC-3′. The other primer was downstream of a natural BamHI site, and thus an 800 bp AflII-BamHI fragment was isolated and ligated between the same sites of pKOS231-114A, which attached the PCR product to most of *srmGIII* down to a BsrGI site (pKOS232-178). The large SpeI to BsrGI fragment of this vector was then ligated to the large BsrGI to AvrII fragment of pKOS231-132, which contained the remainder of *srmGIII,* as well as *srmGIV* and *srmGV*, to give pKOS232-182.

The *chmGI-II* cassette as a NdeI-HindIII fragment (as described in [17]), the *srmGIII-V* cassette as a HindIII-SpeI fragment, and the 8 kb NdeI-SpeI fragment of pKOS232-189 were joined in a three-fragment ligation and recovered by in vitro packaging (Gigapak Gold III, Stratagene). The resulting plasmid, pKOS232-184A, was introduced into *E. coli* DH5α/pUB307 and then into *S. fradiae* K159-1/pKOS244-017A by conjugation.

Analysis of Fermentation Products

Cultures were grown for 7–8 days at 28°C in R media [30]. Culture broth samples were prepared for LC-MS analysis by centrifugation and filtration through 0.2 μm filters or by ethyl acetate extraction, drying, and dissolution of the residue in methanol. Aqueous samples were subjected to on-line solid-phase extraction before switching to the column for fractionation. A Metachem Metasil Basic column (4.6 × 150 mm, 5 μm particle) was used with an 8 min linear gradient from 35% to 100% buffer B (4:1 MeCN:MeOH, 5 mM NH_4Ac) in buffer A (5 mM NH_4Ac in water) at a flow rate of 1 ml/min. There was simultaneous detection by API mass spectrometry (Turbo Ionspray source) and UV absorption at 280 nm.

Isolation and Structural Characterization of Compounds

To isolate 4″-despropionyl-14-methylniddamycin (DPMN), clarified fermentation broth (250 ml) was adjusted to pH 9.5 with 1 N NaOH and extracted with $CHCl_3$ (3 × 300 ml). The organic extracts were combined, dried over Na_2SO_4, filtered, and concentrated in vacuo to provide an amber oil (~1 g). The oil was applied to a silica flash column and eluted with a gradient of 25%–55% acetone/hexane (+ 2% NEt_3). Fractions eluting in 40% acetone/hexane (+2% NEt_3) were pooled and concentrated in vacuo to provide a solid that was dissolved in a minimum amount of CH_2Cl_2 and precipitated with hexane. The solvent was removed in vacuo to provide DPMN (0.20 g, 0.283 mmol) as a white powder. ^{1}H NMR ($CDCl_3$, 400 MHz) δ ppm 1.06 (d, 3H, *J* = 6.4 Hz), 1.19 (m, 9H), 1.24 (m, 6H), 1.48 (m, 1H), 1.56 (m, 1H), 1.72 (d, 1H, *J* = 3.4 Hz), 1.85 (m, 1H), 1.99 (d, 1H, *J* = 14.4 Hz), 2.22 (m, 2H), 2.40 (m, 2H), 2.44 (s, 6H), 2.50 (m, 1H), 2.70 (m, 2H), 2.90 (m, 1H), 3.09 (d, 1H, *J* = 10.0 Hz), 3.23 (m, 2H), 3.48 (dd, 1H, *J* = 7.6, 10.4 Hz), 3.51 (s, 3H), 3.75 (d, 1H, *J* = 10.0 Hz), 4.02 (m, 2H), 4.37 (s, 1H, *J* = 7.6, 10.4 Hz), 4.82 (dquart, 1H, *J* = 10.0, 6.4 Hz), 5.03 (d, 1H, *J* = 3.4 Hz), 5.85 (dd, 1H, *J* = 14.8, 10.0 Hz), 6.11 (dd, 1H, *J* = 14.8, 11.2 Hz), 6.28 (d, 1H, *J* = 14.8 Hz), 7.19 (dd, 1H, *J* = 14.8, 11.2 Hz), 9.37 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm 15.87, 17.29, 17.88, 18.16, 18.94, 25.31, 31.89, 32.32, 38.03, 40.81, 41.88, 43.21, 44.67, 45.75, 61.81, 65.93, 67.58, 68.70, 69.35, 71.70, 73.05, 74.95, 73.05, 76.33, 79.16, 85.28, 96.39, 103.94, 122.29, 129.41, 143.36, 147.74, 173.22, 202.35, 202.45; HRMS calc. for $C_{36}H_{60}NO_{13}$ $[MH]^+$: 714.40592; found: 714.40808.

To isolate 6-carboxymethyl-6-desethyl-14-methyl-5-*O*-mycaminosylplatenolide (CDMP), clarified fermentation broth (1 liter) was applied to a column of XAD-16 resin (500 ml). The column was eluted with water (3.5 liter), removing most of the yellow material, and then with 33%, 50%, and 100% MeOH/H_2O. Fractions eluting with 100% MeOH were pooled, and solvent was removed in vacuo to provide a brown oil (~920 mg). The oil was applied to a silica flash column and first eluted with 10%–100% acetone/hexane (+2% NEt_3) to remove nonpolar material, followed by 0%–10% MeOH/CH_2Cl_2 (+2% NEt_3). Fractions eluting in 10% MeOH/CH_2Cl_2 (+2% NEt_3) were pooled and concentrated in vacuo to provide a yellow oil. The material was applied to a second silica flash column, eluting with 0%–20% MeOH/CH_2Cl_2 (+2% NEt_3). Fractions eluting in 5%–10% MeOH/CH_2Cl_2 (+2% NEt_3) were pooled and concentrated in vacuo to provide a yellow solid (~40 mg). This material was subjected to HPLC (150 × 212 mm 5 μ MetaChem Polaris C-18 column, 10 ml/min), eluting with a gradient of 30%–100% A/B (A = 5 mM NH_4OAc CH_3CN:MeOH [4:1]; B = 5 mM NH_4OAc). Fractions eluting in 45% A/B were pooled and concentrated in vacuo. Residual NH_4OAc was removed by application of the material to a silica flash plug and elution with 10% MeOH/CH_2Cl_2 (+2% NEt_3). The product was concentrated in vacuo to provide CDMP (0.018 g, 0.031 mmol) as a white solid. ^{1}H NMR (CD_3OD, 400 MHz) δ ppm 1.09 (d, 3H, *J* = 6.4 Hz), 1.18 (d, 3H, *J* = 7.2 Hz), 1.30 (m, 6H), 1.67 (m, 3H), 2.29 (m, 2H), 2.37 (d, 1H, *J* = 15.6 Hz), 2.56 (m, 3H), 2.95 (s, 6H), 3.11 (t, 1H, *J* = 10.0 Hz), 3.25 (d, 1H, *J* = 10.6 Hz), 3.31 (m, 1H), 3.36 (m, 1H), 3.52 (m, 1H), 3.55 (s, 3H), 3.88 (d, 1H, *J* = 10.6 Hz), 4.03 (d, 1H, *J* = 9.6 Hz), 4.52 (d, 1H, *J* = 7.2 Hz), 4.78 (dquart, 1H, *J* = 10.0, 6.4 Hz),

5.91 (dd, 1H, J = 14.8, 10.0 Hz), 6.31 (dd, 1H, J = 14.8, 11.2 Hz), 6.57 (d, 1H, J = 14.8 Hz), 7.19 (dd, 1H, J = 14.8, 11.2 Hz); ^{13}C NMR (100 MHz, CD_3OD) δ ppm 14.79, 16.38, 16.42, 16.87, 41.88, 32.06, 33.64, 34.83, 38.29, 40.78, 44.58, 45.16, 60.89, 67.80, 68.68, 69.61, 70.91, 72.67, 72.74, 79.33, 85.66, 102.99, 122.75, 129.76, 143.58, 147.78, 171.99, 178.24, 204.49; HRMS calc. for $C_{29}H_{48}NO_{11}$ $[MH]^+$: 586.32219; found: 586.32024.

To purify 12,16-didesmethyl-DMT, clarified fermentation broth (1.15 liter) was adjusted to pH 7.8 with $NaHCO_3$, the solution filtered, and the filtrate extracted with CH_2Cl_2 (4 × 850 ml). The combined organic extracts were dried over Na_2SO_4, filtered, and concentrated in vacuo to provide a yellow semisolid (377 mg). This material was applied to a silica flash column, eluting with 10%–60% acetone/hexane (+2% NEt_3). Fractions eluting in 30% acetone/hexane (+2% NEt_3) were pooled and solvent was removed in vacuo to provide an off-white solid (73 mg). This material was subjected to HPLC (150 × 212 mm 5 μ MetaChem Polaris C-18 column, 10 ml/min), eluting with a gradient of 50%–100% A/B (A = 5 mM NH_4OAc CH_3CN:MeOH [4:1]; B = 5 mM NH_4OAc). Fractions eluting between 20 and 25 min were pooled and partitioned between CH_2Cl_2 and aqueous saturated $NaHCO_3$ to remove residual NH_4OAc. The organic extracts were combined, dried over Na_2SO_4, and filtered. Hexane was added until the solution became cloudy and solvent was removed in vacuo to provide a white solid (25.5 mg). The material was subjected to a second round of HPLC, as just described, to provide 12,16-didesmethyl-DMT (~12 mg) as a white powder. The structure was verified by ^{1}H, ^{13}C, COSY, multiplicity edited HSQC, and HMBC. ^{1}H NMR ($CDCl_3$, 400 MHz), δ ppm 9.65 (s, 1H, 19-CHO), 7.26 (dd, 1H, 11-H, $J_{11\text{-}10}$ = 14.8 Hz, $J_{11\text{-}12}$ = 14.8 Hz), 6.28 (d, 1H, 10-H, $J_{10\text{-}11}$ = 15.2 Hz), 6.18 (dd, 1H, 12-H), 6.15 (dd, 1H, 13-H), 5.10 (dq, 1H, 15-H, $J_{15\text{-}14}$ = 10 Hz, $J_{15\text{-}16}$ = 6.4 Hz), 5.00 (d, 1H, 1″-H, $J_{1''\text{-}2''}$ = 3.2 Hz), 4.20 (d, 1H, 1′-H, $J_{1'\text{-}2'}$ = 7.2 Hz), 4.05 (dq, 1H, 5″-H, J = 9.6 Hz & 6 Hz), 3.84 (d, 1H, 3-H, $J_{3\text{-}2}$ = 10 Hz), 3.74 (d, 2H, 21-H, $J_{21\text{-}14}$ = 4.8 Hz), 3.70 (d, 1H, 5-H, $J_{5\text{-}4}$ = 9.2 Hz), 3.52 (dd, 1H, 2′-H, $J_{2'\text{-}1'}$ = 7.6 Hz, $J_{2'\text{-}3'}$ = 10.2 Hz), 3.24 (1H, 4′-H, overlapped with 5′), 3.24 (1H, 5′-H, overlapped with 4′), 2.93 (d, 1H, 4″-H, $J_{4''\text{-}5''}$ = 10 Hz), 2.87 (dd, 1H, 18a-H, $J_{18a\text{-}18b}$ = 18 Hz, $J_{18\text{-}19}$ = 8.4 Hz), 2.56 (m, 1H, 8-H), 2.56 (1H, 2a-H, overlapped with 8-H), 2.47 (s, 6H, 3′-NMe_2), 2.46 (1H, H-3′, overlapped with 3′-NMe_2), 2.35 (m, 1H, 14-H), 2.04 (dd, 1H, 2″a-H), 2.00 (dd, 1H, 2b-H), 2.00 (dd, 1H, 2″-a), 1.74 (dd, 1H, 2″b-H, $J_{2''a\text{-}2''b}$ = 14.4, Hz $J_{1''\text{-}2''}$ = 3.6 Hz), 1.68 (m, 1H, 4-H), 1.59 (m, 1H, 6-H), 1.32 (d, 3H, 16-H, $J_{16\text{-}15}$ = 6.4 Hz), 1.27 (d, 3H, 6″-H, $J_{6''\text{-}5''}$ = 6.4 Hz), 1.24 (m, 2H, 7-H), 1.23 (d, 3H, 6′-H, $J_{6'\text{-}5'}$ = 7.6 Hz), 1.22 (s, 3H, 3″-Me), 1.17 (d, 3H, 20-H, $J_{20\text{-}8}$ = 6.8 Hz), 1.00 (d, 3H, 17-H, $J_{17\text{-}4}$ = 6.8 Hz) . ^{13}C NMR ($CDCl_3$, 100 MHz), δ ppm 203.0 (C-9), 202.7 (C-19), 173.4 (C-1), 143.5 (C-13), 143.1 (C-11), 131.8 (C-12), 122.6 (C-10), 103.6 (C-1′), 96.4 (C-1″), 80.8 (C-5), 76.3 (C-4″), 74.9 (C-4′), 73.1 (C-5′), 71.7 (C-2′), 69.5 (C-15), 69.4 (C-3″), 69.4 (C-3), 68.6 (C-3′), 65.9 (C-5″), 61.8 (C-21), 53.5 (C14), 44.6 (C-8), 43.4 (C-18), 41.9 (3′-NMe_2), 40.8 (C-2″), 39.8 (C-2), 32.1 (C-4), 31.8 (C-6), 29.6 (C-7), 25.3 (C-3″-Me), 18.9 (C-6′), 18.3 (C-16), 18.2 (C-6″), 17.3 (C-20), 9.0 (C-17). HRMS calc. for $C_{36}H_{60}NO_{13}$ $[MH]^+$: 714.40592, found: 714.40457.

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References

1. Katz, L. (1997). Manipulation of modular polyketide synthases. Chem. Rev. *97*, 2557–2576.
2. Ikeda, H., and Omura, S. (2002). Biosynthesis, regulation, and genetics of macrolide production. In Macrolide Antibiotics: Chemistry, Biology, and Practice, Second Edition, S. Omura, ed. (New York: Academic Press), pp. 285–326.
3. Baltz, R.H., and Seno, E.T. (1981). Properties of *Streptomyces fradiae* mutants blocked in biosynthesis of the macrolide antibiotic tylosin. Antimicrob. Agents Chemother. *20*, 214–225.
4. Baltz, R.H., Seno, E.T., Stonesifer, J., and Wild, G.M. (1983). Biosynthesis of the macrolide antibiotic tylosin. A preferred pathway from tylactone to tylosin. J. Antibiot. (Tokyo) *36*, 131–141.
5. Cox, K.L., Fishman, S.E., Larson, J.L., Stanzak, R., Reynolds, P.A., Yeh, W.K., van Frank, R.M., Birmingham, V.A., Hershberger, C.L., and Seno, E.T. (1986). The use of recombinant DNA techniques to study tylosin biosynthesis and resistance in *Streptomyces fradiae*. J. Nat. Prod. *49*, 971–980.
6. Fishman, S.E., Cox, K., Larson, J.L., Reynolds, P.A., Seno, E.T., Yeh, W.K., Van Frank, R., and Hershberger, C.L. (1987). Cloning genes for the biosynthesis of a macrolide antibiotic. Proc. Natl. Acad. Sci. USA *84*, 8248–8252.
7. Baltz, R.H., and Seno, E.T. (1988). Genetics of *Streptomyces fradiae* and tylosin biosynthesis. Annu. Rev. Microbiol. *42*, 547–574.
8. Merson-Davies, L.A., and Cundliffe, E. (1994). Analysis of five tylosin biosynthetic genes from the tylIBA region of the *Streptomyces fradiae* genome. Mol. Microbiol. *13*, 349–355.
9. Gandecha, A.R., Large, S.L., and Cundliffe, E. (1997). Analysis of four tylosin biosynthetic genes from the tylLM region of the *Streptomyces fradiae* genome. Gene *184*, 197–203.
10. Bate, N., and Cundliffe, E. (1999). The mycinose-biosynthetic genes of *Streptomyces fradiae*, producer of tylosin. J. Ind. Microbiol. Biotechnol. *23*, 118–122.
11. Cundliffe, E. (1999). Organization and control of the tylosin-biosynthetic genes of *Streptomyces fradiae*. Actinomycetologica *13*, 68–75.
12. Fouces, R., Mellado, E., Diez, B., and Barredo, J.L. (1999). The tylosin biosynthetic cluster from *Streptomyces fradiae*: genetic organization of the left region. Microbiol. *145*, 855–868.
13. Bate, N., Butler, A.R., Smith, I.P., and Cundliffe, E. (2000). The mycarose-biosynthetic genes of *Streptomyces fradiae*, producer of tylosin. Microbiol. *146*, 139–146.
14. Cundliffe, E., Bate, N., Butler, A., Fish, S., Gandecha, A., and Merson-Davies, L. (2001). The tylosin-biosynthetic genes of Streptomyces fradiae. Antonie Van Leeuwenhoek *79*, 229–234.
15. Richardson, M.A., Kuhstoss, S., Huber, M.L., Ford, L., Godfrey, O., Turner, J.R., and Rao, R.N. (1990). Cloning of spiramycin biosynthetic genes and their use in constructing *Streptomyces ambofaciens* mutants defective in spiramycin biosynthesis. J. Bacteriol. *172*, 3790–3798.
16. Kuhstoss, S., Huber, M., Turner, J.R., Paschal, J.W., and Rao, R.N. (1996). Production of a novel polyketide through the construction of a hybrid polyketide synthase. Gene *183*, 231–236.
17. Ward, S.L., Hu, Z., Schirmer, A., Reid, R., Revill, W.P., Reeves, C.D., Petrakovsky, O.V., Dong, S.D., and Katz, L. (2004). The chalcomycin biosynthesis gene cluster from *Streptomyces bikiniensis*: novel features of an unusual ketolide through expression of the *chm* PKS in *Streptomyces fradiae*. Antimicrob. Agents Chemother., in press.
18. Pernodet, J.L., Boccard, F., Alegre, M.T., Blondelet-Rouault, M.H., and Guerineau, M. (1988). Resistance to macrolides, lincosamides and streptogramin type B antibiotics due to a mutation in an rRNA operon of *Streptomyces ambofaciens*. EMBO J. *7*, 277–282.
19. Rodriguez-Fonseca, C., Amils, R., and Garrett, R.A. (1995). Fine structure of the peptidyl transferase centre on 23 S-like rRNAs deduced from chemical probing of antibiotic-ribosome complexes. J. Mol. Biol. *247*, 224–235.
20. Schlunzen, F., Zarivach, R., Harms, J., Bashan, A., Tocilj, A., Albrecht, R., Yonath, A., and Franceschi, F. (2001). Structural basis for the interaction of antibiotics with the peptidyl transferase centre in eubacteria. Nature *413*, 814–821.
21. Hansen, J.L., Ippolito, J.A., Ban, N., Nissen, P., Moore, P.B., and Steitz, T.A. (2002). The structures of four macrolide antibiotics bound to the large ribosomal subunit. Mol. Cell *10*, 117–128.
22. Liu, M., and Douthwaite, S. (2002). Methylation at nucleotide G745 or G748 in 23S rRNA distinguishes Gram-negative from Gram-positive bacteria. Mol. Microbiol. *44*, 195–204.
23. Liu, M., and Douthwaite, S. (2002). Resistance to the macrolide antibiotic tylosin is conferred by single methylations at 23S rRNA nucleotides G748 and A2058 acting in synergy. Proc. Natl. Acad. Sci. USA *99*, 14658–14663.
24. Lebars, I., Yoshizawa, S., Stenholm, A.R., Guittet, E., Douthwaite, S., and Fourmy, D. (2003). Structure of 23S rRNA hairpin 35 and its interaction with the tylosin-resistance methyltransferase RlmAII. EMBO J. *22*, 183–192.

25. Poehlsgaard, J., and Douthwaite, S. (2003). Macrolide antibiotic interaction and resistance on the bacterial ribosome. Curr. Opin. Investig. Drugs *4*, 140–148.
26. Schlunzen, F., Harms, J.M., Franceschi, F., Hansen, H.A., Bartels, H., Zarivach, R., and Yonath, A. (2003). Structural basis for the antibiotic activity of ketolides and azalides. Structure (Camb) *11*, 329–338.
27. Liu, M., Kirpekar, F., Van Wezel, G.P., and Douthwaite, S. (2000). The tylosin resistance gene tlrB of *Streptomyces fradiae* encodes a methyltransferase that targets G748 in 23S rRNA. Mol. Microbiol. *37*, 811–820.
28. Kiyoshima, K., Sakamoto, M., Nomura, H., Yoshioka, T., Okamoto, R., Sawa, T., Naganawa, H., and Takeuchi, T. (1989). Structure-activity relationship studies on 4″-O-acyltylosin derivatives: significance of their 23-O-mycinosyl and 4″-O-acyl moieties in antimicrobial activity against macrolide-resistant microbes. J. Antibiot. (Tokyo) *42*, 1661–1672.
29. Zhanel, G.G., Walters, M., Noreddin, A., Vercaigne, L.M., Wierzbowski, A., Embil, J.M., Gin, A.S., Douthwaite, S., and Hoban, D.J. (2002). The ketolides: a critical review. Drugs *62*, 1771–1804.
30. Rodriguez, E., Hu, Z., Ou, S., Volchegursky, Y., Hutchinson, C.R., and McDaniel, R. (2003). Rapid engineering of polyketide overproduction by gene transfer to industrially optimized strains. J. Ind. Microbiol. Biotechnol. *30*, 480–488.
31. Rodriguez, E., Ward, S.L., Fu, H., Revill, W.P., McDaniel, R., and Katz, L. (2004). Engineered biosynthesis of 16-membered macrolides that require methoxymalonyl-ACP precursors in *Streptomyces fradiae*. Appl. Microbiol. Biotechnol., in press.
32. Broadhurst, R.W., Nietlispach, D., Wheatcroft, M.P., Leadlay, P.F., and Weissman, K.J. (2003). The structure of docking domains in modular polyketide synthases. Chem. Biol. *10*, 723–731.
33. Gregory, M.A., Till, R., and Smith, M.C. (2003). Integration site for *Streptomyces* phage phiBT1 and development of site-specific integrating vectors. J. Bacteriol. *185*, 5320–5323.
34. Woo, P.W.K., Dion, H.W., and Bartz, Q.R. (1964). The structure of chalcomycin. J. Am. Chem. Soc. *86*, 2726–2727.
35. Huang, S.L., Hassell, T.C., and Yeh, W.K. (1993). Purification and properties of NADPH-dependent tylosin reductase from *Streptomyces fradiae*. J. Biol. Chem. *268*, 18987–18993.
36. Jacobsen, J.R., Hutchinson, C.R., Cane, D.E., and Khosla, C. (1997). Precursor-directed biosynthesis of erythromycin analogs by an engineered polyketide synthase. Science *277*, 367–369.
37. Kieser, T., Bibb, M.J., Buttner, M.J., Chater, K.F., and Hopwood, D.A. (2000). Practical Streptomyces Genetics (Norwich, UK: The John Innes Foundation).
38. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).
39. Bierman, M., Logan, R., O'Brien, K., Seno, E.T., Rao, R.N., and Schoner, B.E. (1992). Plasmid cloning vectors for the conjugal transfer of DNA from *Escherichia coli* to *Streptomyces* spp. Gene *116*, 43–49.